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Dose Range Finding of Chromophore Powder: A Summary Report

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

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FOR THE DIRECTOR

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MARK M. HOFFMAN
Deputy Chief, Biosciences and Protection Division
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The potential toxic effects of chromophore powder in primary hepatocytes were investigated. Initially, a solubility assessment was conducted in aqueous and organic solutions. The solubility test showed that the chromophore powder is soluble in DMSO at 0.001 mg/ml, while not in water or ethyl alcohol. Therefore, DMSO was selected as the solvent for the dose range finding study. For initial toxicity evaluations, mitochondrial function (MTT assay), lactate dehydrogenase release (LDH assay) and the morphology of cells were assessed for primary hepatocytes under control or exposed conditions (2 hour treatment followed by 24 h of incubation in fresh media). The microscopic studies demonstrated that chromophore-exposed cells at the doses of 0.5 and 1 mg/ml became abnormal in size displaying shrinkage and irregular shape. The results showed that chromophore powder had no measurable effect on mitochondrial function. However, LDH assay showed a significant increase in LDH release at 0.1mg/ml, while there was no change at 0.5 and 1.0 mg/ml. The dose range finding study indicates that chromophore powder displayed limited toxicity in vitro at increasing dose.

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Summary

The potential toxic effects of chromophore powder in primary hepatocytes were investigated. Initially, a solubility assessment was conducted in aqueous and organic solutions. The solubility test showed that the chromophore powder was soluble in DMSO at 0.001 mg/ml. Unlike all preparations at higher concentrations as well as solutions prepared with water or ethanol, this chromophore powder preparation was very clear and without insolublized particles. Therefore, DMSO was selected as the solvent for this toxic dose range finding study. For initial toxicity evaluations, mitochondrial function (MTT assay), lactate dehydrogenase release (LDH assay) and the morphology of cells were assessed for primary hepatocytes under control (DMSO only) or exposed conditions (2 hour treatment followed by 24 h of incubation in fresh media). The microscopic studies demonstrated that chromophore-exposed cells at the doses of 0.5 and 1 mg/ml media became abnormal in size displaying shrinkage and irregular shape. The results showed that chromophore powder had no measurable effect on mitochondrial function. However, LDH assay showed a significant increase in LDH released into the medium at 0.1mg/ml, while there was no change at 0.5 and 1.0 mg/ml. The dose range finding study indicates that chromophore powder displayed limited toxicity *in vitro* at increasing dose.

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TABLE OF CONTENTS

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1.0. Introduction

The Biotechnology Group in the Materials and Manufacturing Laboratory, Directorate of the Air Force Research Laboratory (AFRL/ML) provided chromophore powder to the Operational Toxicology Branch, in the Human Effectiveness Directorate (AFRL/HEST). The chromophore is biologically derived and possesses desirable spectral properties for defense applications. The fundamental structure of the chromophore powder is porphyrin-based and the powder consists primarily of a formaldehyde cross-linked preparation of bacterial cell mass. Before additional test and evaluation commenced, a general acute toxicological assessment was performed to establish a baseline toxicity measurement and for selection of further studies to determine exposure risks for personnel. In deployment scenarios, there is potential concern over possible dermal or inhalation exposures. A series of preliminary, in vitro toxicology assessments are routinely performed by AFRL/HEST as described here.

2. 0. Materials and Methods

2. 1. Chemicals. The test material (chromophore powder) received from AFRL/ML was a newly synthesized chemical and no details on the chemical properties were available. The molecular weight was also not available for this newly synthesized material, therefore concentration of the test material solutions were expressed as mg/ml rather than millimoles. Collagenase was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-nicotinamide-adenine dinucleotide-reduced (NADH), 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), reduced glutathione (GSH), insulin/transferrin/sodium selenite (ITS) additive, gentamycin, and dexamethasone were purchased from Sigma Chemical Company (St. Louis, MO). Chee media was obtained from Gibco (Grand Island, NY).

2.2. Animals. Male Fischer 344 rats (225-300 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with 1 ml/kg of a mixture of

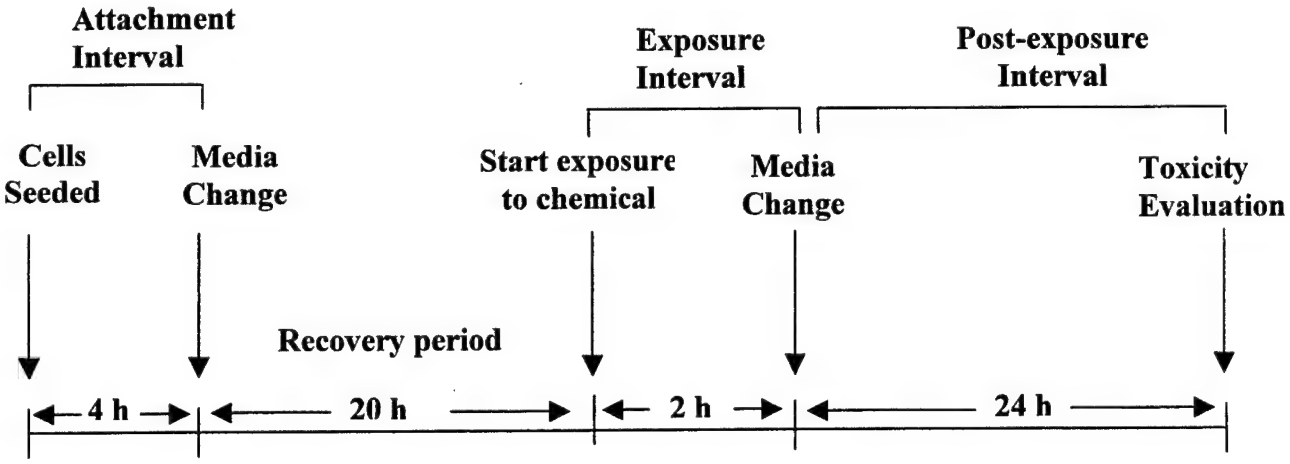
ketamine (70 mg/L; Parke-Davis, Moris Plains, NJ) and xylazine (6 mg/L; Mobay Corp., Shawnee, KS) prior to undergoing liver perfusion. All animals used in this study were handled in accordance with the principles stated in *Guide for the Care and Use of Laboratory Animals*, (National Research Council, National Academy Press, 1996) and the Animal Welfare Act of 1966, as amended.

2.3. Liver Perfusion, Hepatocytes Enrichment and Culture. Following perfusion of rat liver, hepatocytes were isolated and enriched by the two step Seglen procedure (Seglen, 1976) with minor modifications as previously described (Hussain and Frazier 2002). Cells were seeded in either 96-well (4×10^4 cells/well) or 6-well (1.0×10^6 cells/well) culture plates previously coated with rat tail collagen, 1.0 μg/well or 25 μg/well respectively. Following attachment, hepatocytes were cultured for an additional 20 h before treatment (Figure 1).

2.4. Solubility Tests: The solubility test was performed in deionized water, ethyl alcohol and dimethyl sulfoxide (DMSO). After examining solubility, DMSO solvent was used to prepare stock solutions of the chromophore powder for toxicity testing.

2.5. Treatment and Toxicity Endpoints: Primary rat hepatocytes were treated with a range of concentrations of chromophore powder dissolved in Chee culture media (100 ul in 96 well plates and 1 ml in 6 well plates) and exposed for 2 h (Figure 1). At the end of the 2 h incubation period, the media was replaced with fresh media and further incubated for 24 h. After 24 h postincubation, the following toxicity end points (LDH, MTT and external morphology) were evaluated.

Figure 1



2.5.1. LDH Leakage: Lactate dehydrogenase (LDH) leakage indicates loss of cellular viability because membrane damage that results in LDH leakage is generally considered irreversible (Moldeus et al. 1978). LDH leakage was assessed by spectrophotometrically measuring the oxidation of NADH at 340 nm in both the cells and media as described by Moldeus et al., (1978). The total release of activity (percentage) in the media was then calculated by dividing the amount of activity in the media by the total activity (medium plus cell lysate).

2.5.2. Mitochondrial Function: Mitochondrial function was determined spectrophotometrically by measuring the degree of mitochondrial reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by succinic dehydrogenase (Carmichael et al., 1987; Hussain and Frazier 2002).

2.5.3 Qualitative observation of external morphology of control and exposed cells by phase contrast inverted microscopy: Cells (1×10^6) were exposed as mentioned above at various concentrations of chromophore for 2 h and then the medium was replaced and cells incubated for an additional 24 h. After completion of the incubation period, hepatocytes were washed with PBS and observed by phase contrast inverted microscopy at 100x magnification.

2.6. Statistical Evaluation: The data were expressed as mean ± standard deviation (SD) of three independent experiments with hepatocytes from three different rats. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett's Method for multiple comparisons. A value of $p < 0.05$ was considered significant. SigmaStat for Windows version 2.03 software was used for the statistical analysis.

3.0. Results

3.1. Physical observation on solubility test of chromophore

Table 1 shows the degree of solubility for different concentrations of chromophore powder in DMSO, ethyl alcohol and deionized water. The solubility ranges were minimal (from very light to white powder suspension) at 0.001-1.00 mg/ml of chromophore powder. The white suspension of chromophore powder precipitates above 0.001 mg/ml in all solvents tested indicating that maximum solubility occurred well below 1 mg/ml. Therefore toxicity testing between 0.001 and 1 mg/ml contain minor to significant solid precipitant. The degree of solubility of chromophore powder follows: DMSO > ethyl alcohol > deionized water. DMSO was used as solvent of choice in the study as the suspension was very clear when compared with the other two solvents.

Table 1: Solubility Assessment

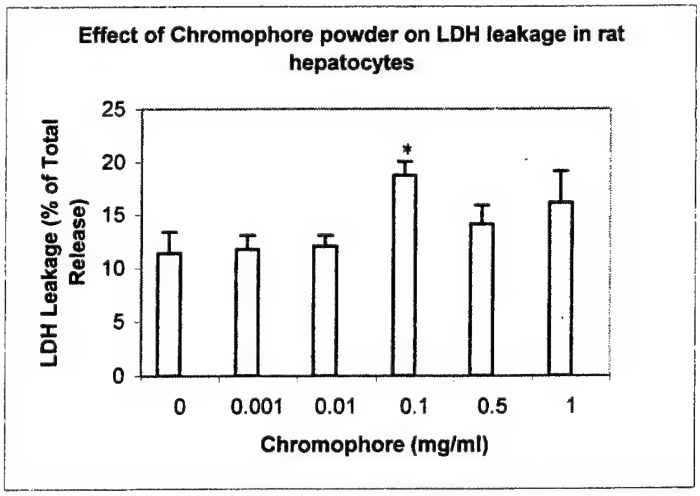
Solvents	Chromophore Powder Concentration			
	1 mg/ml	**0.1 mg/ml**	**0.01 mg/ml**	**0.001 mg/ml**
DMSO	White suspension	Light white suspension	Light white suspension	Very light suspension (Very Clear)
Ethyl alcohol	White-suspension	Light white suspension	Light white suspension	Very light suspension (Clear)
Deionized water	White-suspension-with few particles	White-suspension-with few particles	White-suspension-	Very light suspension

3.2. Cytotoxicity of Chromophore powder

A toxic dose range finding study was conducted to determine the cytotoxicity resulting from chromophore exposure. In these studies, hepatocytes were exposed to chromophore powder at concentration ranges from 0.001-1.00 mg/ml for 2 h. At the end of the 2 h incubation period, the media was replaced with fresh media and cells were further incubated for 24 h. After 24 h postincubation, cytotoxicity was evaluated using LDH leakage and MTT reduction as endpoints. The qualitative external morphology of control and exposed cells by phase contrast inverted microscopy was also observed.

Membrane damage that results in LDH leakage is generally considered irreversible; therefore, LDH leakage was selected as a biomarker for cellular viability (Hussain et al., 2001). The results for LDH leakage indicate that a 0.001 or 0.01 mg/ml chromophore exposure did not produce cytotoxicity while the 0.1 mg/ml exposure exhibited significant cytotoxicity. However, there was no statistical change in toxicity at 0.5 or 1 mg/ml doses (Figure 2).

Figure 2



Mitochondria are vulnerable targets for toxic injury by a variety of compounds because of their crucial role in maintaining cellular structure and function via aerobic ATP production (Hussain et al., 2002). Therefore, the MTT assay that represents mitochondrial function was selected as a biomarker to assess if the chromophore affects mitochondrial function. The results of MTT assays showed that the chromophore did not produce cytotoxicity at all doses tested (Figure 3). There was a slight increase in mitochondrial function at the 0.001 and 0.01 mg/ml doses of chromophore powder, but these were not statistical significant. There was no change in MTT function at 0.1-1 mg/ml indicating that the mitochondrial function was not changed at these doses.

Figure 3

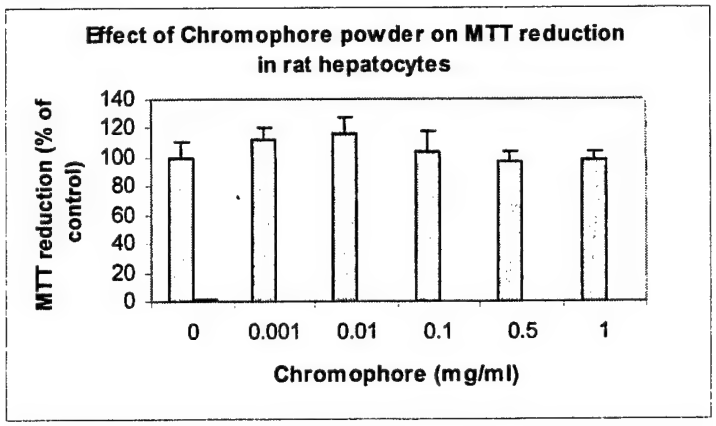
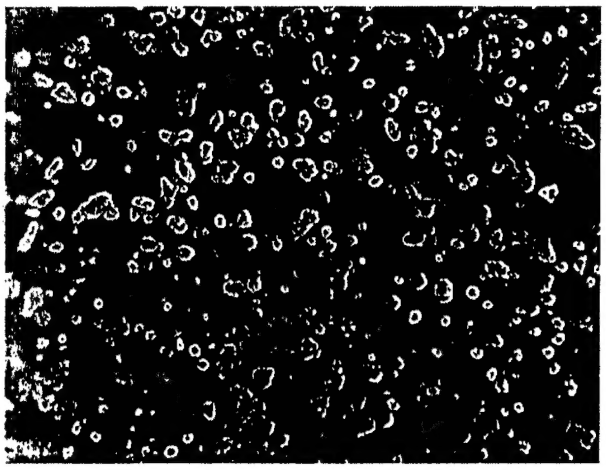


Figure 4 shows the general external morphology of control and chromophore exposed cells. The chromophore particles associated with cells appeared in black color when observed under microscope. These results show a dose dependent change in the shape of the cells. At the low dose of 0.01 mg/ml, the cells appears to be close to control with few black particles associated with the cells are probably chromophore particles adhering to the cell membrane. With increasing dose, cells started to shrink and became irregular in shape and associated chromophore particles increased (Figures 4 C-F). The microscopic results indicate that not all chromophore particles were accumulated into the cells, rather some associated with membranes.

Figure 4. Qualitative observation of external morphology of control and exposed cells by phase contrast inverted microscopy. A Control; B 0.001 mg/ml; C 0.01 mg/ml; D 0.1 mg/ml; E 0.5 mg/ml; F 1 mg/ml.

A



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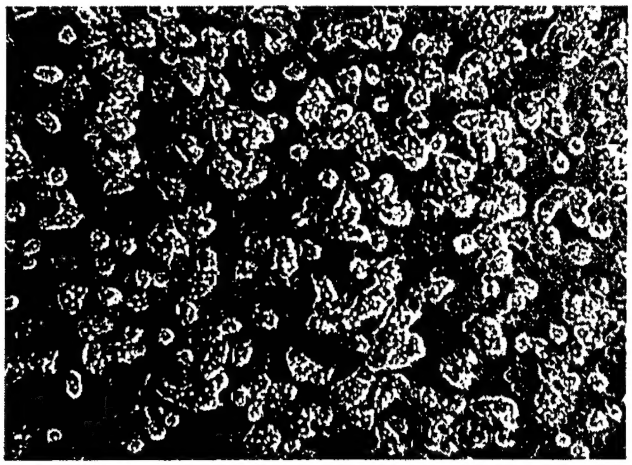

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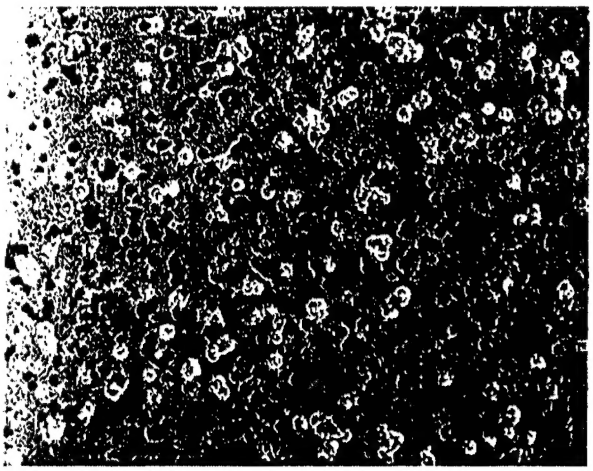

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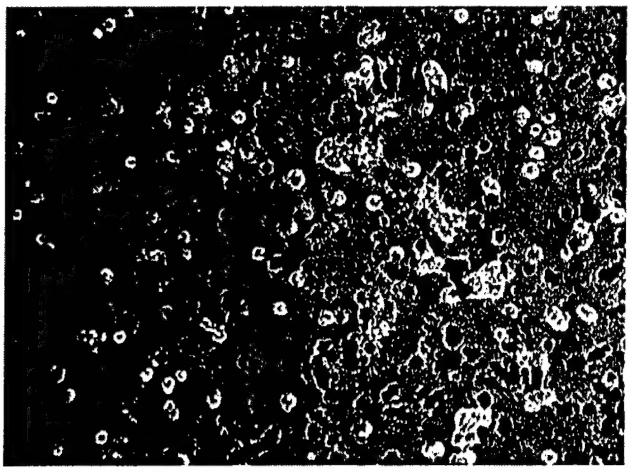

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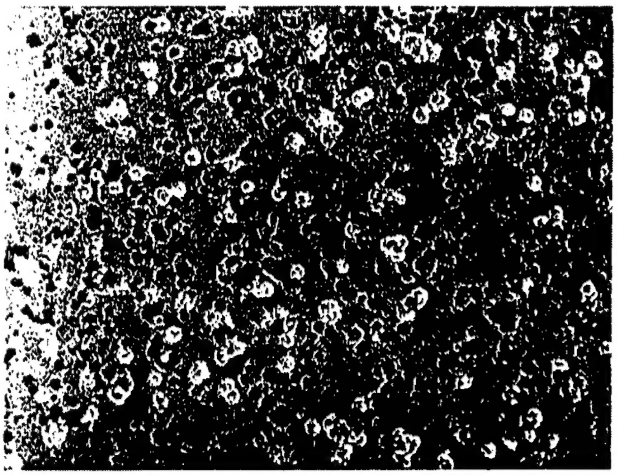

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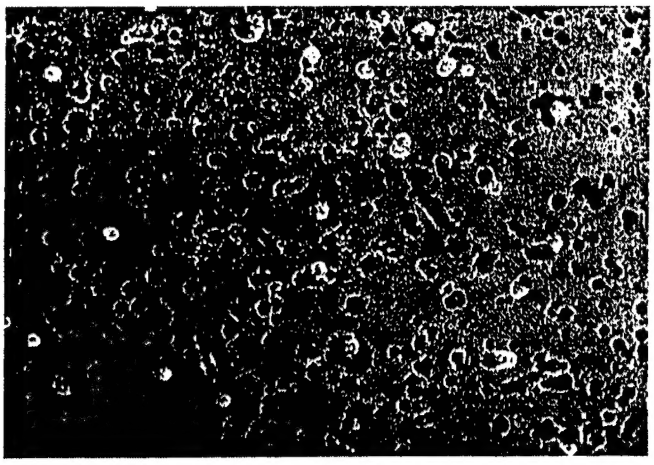

3.3. Validation Results with Reference Chemicals

In order to validate the assay system, we compared results of chromophore powder with other known chemicals such as Cadmium (Cd), hydrazinium nitrate (HZN), and 1,2,4-triazole nitrate (TN). The EC_{50} values of these compounds are shown in Table 2. Due to solubility problems 1mg/ml of chromophore powder was the maximum amount for toxicity testing. On the basis of these results, the estimated approximate EC_{50} value for chromophore toxicity is above 1 mg/ml (Table 3).

Table 2: Calculated EC_{50} values of reference chemicals

Chemical	MTT EC_{50}	LDH EC_{50}
Cd	1.38 µg/ml	0.45 µg/ml
HZN	6.2 mg/ml	4.75 mg/ml
TN	>19.8 mg/ml	>19.8 mg/ml

Cd: Cadmium, HZN: Hydrazinium nitrate, TN: 1,2,4-Triazole nitrate

Table 3: Approximate EC_{50} values of Chromophore compound

Chemical	MTT EC_{50}	LDH EC_{50}
Chromophore	>1.00 mg/ml	>1.00 mg/ml

4.0. Discussion

The chromophore powder is biologically derived and possesses desirable spectral properties for defense applications. The fundamental structure of the chromophore powder is porphyrin-based and the powder consists primarily of a formaldehyde cross-linked preparation of bacterial cell mass. Toxicological evaluation was performed to determine potential acute cellular toxicity of chromophore powder. Since it is a new

compound little information on its chemical structure or analysis is available. Therefore, a series of preliminary, in vitro toxicology assays were performed in rat hepatocytes. The preliminary results showed that there was a slight increase in mitochondrial function at 0.001 or 0.01 mg/ml with no change at 0.1-1mg/ml. The slight increase could be a stimulatory effect at lower dose of the chemical on the mitochondrial function. Overall, there was no statistical change in mitochondrial function (Figure 3). However, there was a statistically significant increase in LDH leakage at 0.1 mg/ml while there was no change at 0.5 or 1 mg/ml (Figure 2). The toxic action of chromophore as evaluated by LDH leakage did not follow the customary dose dependent pattern for this compound. The reason is not known for this response but it could be due to a different pattern of solid compound accumulation. The microscopic results indicate some chromophore particles were not accumulated into the cells but associated with membranes (Figure 4 A-F). The associated particles may have detrimental effects on cells if the experiment were to be extended for a longer period of time. Since we used primary hepatocytes, which do not proliferate for continuous growth, the long-term culture was not possible or the assessment of growth inhibition of the cell-associated particles. Overall, based on the results, the chromophore exhibited a limited toxicity in primary hepatocytes as evidenced by leakage of LDH, MTT reduction and morphological changes with the 24 h time period.

Low solubility was the main hindrance for this compound in studying a wide range of dosimetry. The highest concentration completely soluble in DMSO was 0.001 mg/ml. However, the dose range finding study was investigated up to 1 mg/ml to assess both soluble and insoluble affects of the compound. Another challenge was to determine whether the chromophore was accumulating into the cells. The microscopic observation revealed that chromophore particles are significantly associated with cells rather than dispersed throughout the medium and growth substrate. However, it is not known what percentage of chemical had been taken up by the cells to compare its toxicity among different doses. The major toxicity of exposure is due to solid particles this is because of the very limited solubility in water. There appeared to be a strong propensity of particles to adhere to cell membranes such that the material was virtually

extracted from solution on to the outer membrane. One future direction would be to consider cells specific for particulate uptake i.e., macrophages. The biochemical end points we have taken in this study primarily provide information on preliminary screening of acute toxicity. Further studies are required with additional biochemical end points in order to investigate mechanisms of toxicity with respect to possible exposure risks to personnel.

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